# ICSI Manual of Procedure

This manual applies to ICSI production of equine embryos.
For endangered species, zoo and wildlife animals, please get in touch.

**IMPORTANT:**
**IVM, Holding Medium and the IVC Medium must be supplemented with 5 % Serum.**

**Oocyte Holding Medium must be supplemented with 10 %-20 % Serum.**

**We recommend Fetal Bovine Serum from Sigma-Aldrich F2442; as it is already sterile filtered, there is no need to filter again 5 % serum corresponds to 1 ml per 20 ml bottle.**

**The media also contain a synthetic serum replacement.**

**Do never aliquot any media into plastic vials for storage, it will significantly impair embryo development.**

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# Media Products

| Media | REF | Volume |
|---|---|---|
| OPU Medium<br>For Ovum Pick Up | 4.01.500 | 500 ml |
| IVM Medium<br>For in vitro Maturation of Oocytes | 3.03.020 | 20 ml |
| SEMEN WASH Medium<br>For Semen Preparation Non-Capacitating | 3.05.050 | 50 ml |
| ICSI Medium | 3.06.020 | 20 ml |
| One-Step IVC Medium | 3.13.020 | 20 ml |
| IVC Medium 1- Cleavage<br>For Step 1 in vitro Culture | 3.07.020 | 20 ml |
| IVC Medium 2 -Blastocyst<br>For Step 2 in vitro Culture | 3.08.020 | 20 ml |
| Holding Medium | 3.09.020 | 20 ml |
| Stroebech Heavy OIL | 4.09.050 | 50 ml |
| PVP | 4.10.001 | 1 ml |
| Swim Up | 3.11.050 | 50 ml |
| Hyaluronidase | 4.02.005 | 5x1 ml |
| Oocyte HOLDING Medium<br>For holding of Oocytes prior to maturation | 3.12.020 | 20 ml |
| Equine One Step IVC Medium | 3.13.020 | 20 ml |

IMPORTANT
In vitro produced embryos are much more fragile and sensitive to pH and temperature fluctuations and not as robust as in vivo produced (ET) embryo



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# General Information

**The Incubator temperature** should be 38.2 °C.
All heated stages should be set to 35 °C, not more! (Common mistake)

**Incubator gas concentrations:**

- **Maturation (IVM) 5-6 % $CO_2$ humidified atmospheric air (21 % $O_2$)**
- Culture (IVC) 5-6 % $CO_2$ and 6-7 % $O_2$

**The media contain 25-27 mM bicarbonate corresponding to a pH 7.3-7.4 using the above gas concentrations, see appendix 2.**
**Equine embryos require low oxygen conditions for IVC.**

New option for IVC: Use One-step Equine IVC Medium for the entire culture period. No high glucose in step 2. Medium should still be refreshed/changed day 4,5 or 6.
Semen Wash Medium and OPU Medium should **only** be warmed and **must not** be $CO_2$ equilibrated.

***Should your laboratory be situated above sea level, you will need to adjust the $CO_2$/$O_2$ concentrations accordingly***
***- See appendix 3***

## Media dish preparations

**NOTE: overnight holding of oocytes prior to maturation in Oocyte Holding Medium at 22 °C increases blastocyst rates. NB Many plastic vials are toxic!**

**Maturation:** in 500 µl 4WP without oil overlay.

**ICSI:** prepare droplets just prior to performing ICSI and **after** semen preparation.

**IVC:** Culture embryos in media drops of no less than 100 µl with oil overlay, or preferably in 500 µl 4WP with oil overlay.

**Rinse** dishes: **always rinse** oocytes/embryos once in the corresponding final medium in order not to dilute medium that is to be incubated.

When **oil overlay** is used in 4-well plates, 4WP, rotate the lid during incubation. Just one oil drop between the lid and the dish will create a seal, thereby preventing any $CO_2$ equilibration of the media and risking embryo death.

**Drop (100 µl) preparation:** In order to make sure that the drop forms a dome and isn't flat, first make a drop 'footprint' by placing 10 µl drops on the base of the dish and then cover them with plenty of oil. Afterwards, 'top up' the drops under oil with the remaining 90 µl of media per drop.

## Disposables

Use only polystyrene plastics that are embryo-grade quality. Note that cell culture grade is not good enough! Use 4WP with a culture area per well of 1.9 $cm^2$.

*Only use filtered pipette tips to avoid risk of contamination of pipettes, which may be a hidden source of continuous infection.*



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# DAY -1: Maturation IVM

**Oocyte aspiration by OPU or SH collection**

**Media to be used**

OPU Medium, Holding Medium, IVM Medium and/or Oocyte Holding Medium.

Do never hold oocytes in the OPU medium longer than necessary and max 30 minutes.

**In the IVF Laboratory**

- Warm the Holding Medium
- Prepare and pre-equilibrate IVM Medium in 4WP wells
- Equilibrate IVM Medium for rinsing the COC's prior to transfer to the IVM wells/vials

***If the tubes have a bad smell, take off the lid under a laminar flow hood and let them air out 2 hours or overnight***

## Ovum Pick Up (OPU)

Preheat OPU medium to 37 °C.

Make sure that the aspiration tube warmer is **not too hot**, it should not be more than 37 °C, however, it is often much warmer. Place a thermometer inside a tube with oil in the tube warmer to verify the temperature.

Pour OPU content into a filtered dish and wash with OPU Medium until content is clear. Make sure the dish/filter does not dry out at any time.

Search for oocytes and wash them through the Holding Medium to get rid of debris. One time may be enough.

Rinse once in IVM Medium or Oocyte Holding Medium prior to transfer oocytes to the final maturation/holding dish or vial.

Maturation should be performed in the $CO_2$ incubator at 38.2 °C for 24-30 hours.

Oocytes in Oocyte Holding Medium should be kept at 22 °C and can be kept up to 24 hours.

***Oocytes should be surrounded by multiple layers of cumulus cells. If many are naked, adjust vacuum pump pressure and check the lumen size of the aspiration tubes. Using tubes and needles that are too narrow will cause the unintentional and premature denudation of oocytes during OPU. Equally, using tubes that are too wide will run the risk of stripping the oocytes of their cumulus cells too due to the increased turbulence. Equine oocytes cannot be conventionally aspirated, but the follicular cavity must be flushed several times with a 2-way aspiration system.***



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## Slaughterhouse (SH)

Wash and hold the ovaries in a food plastic grade bag in a thermo-container 20-22 °C prior to curettage. The ovaries can be kept up to 24 hours, however, the results are better if the oocytes are held in Oocyte holding medium rather than holding the ovaries.

Aspirate surface follicles and curettage the walls of the emptied follicles to release the oocytes. Slice the deeper-situated follicles with a sterile scalpel.

Collect the contents of each follicle using the curette, and dip into a 50 ml tube containing the preheated OPU medium each time to recover the oocytes.

Pour follicular content into a filtered dish and wash with OPU Medium until content is clear. Make sure the dish/ filter does not dry out at any time.

Search for oocytes and wash them through 4WP wells or 35 mm dishes depending of number of oocytes in the Holding Medium to get rid of all debris.

Rinse once in IVM Medium prior to transferring oocytes into the final maturation dish as quickly as possible. Place the 4-well dish in the $CO_2$ incubator and mature 24-37 hours.

No more than 20 oocytes per vial if oocytes are held prior to maturation.



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# DAY 0: Day of ICSI and Culture (IVC) step 1

**Media to be used**

IVC Medium, ICSI Medium, Holding Medium, Swim Up Medium, Oil, Hyaluronidase and PVP.

Equilibrate in the $CO_2$ incubator IVC Medium 1- Cleavage and Maturation medium (for extended maturation time)

**Media & Dish Preparation**

- Prepare and equilibrate IVM Medium in a 4WP (for oocytes that need longer maturation)
- Prepare and equilibrate One-step IVC Medium or IVC Medium 1-Cleavage in 4WP with oil overlay and 1 well or dish without oil IVC dish without oil for rinse
- Preheat ICSI medium. Preheat Semen Wash medium (if in the incubator keep the lid on the bottle)
- Place and preheat PVP and hyaluronidase on the heated stage in the laminar flow hood

**Matured oocyte preparation – Denudation (removal of cumulus cells)**

- After 22-24 hours of maturation note maturation by the expansion of the cumulus cells.
- Immediately prior to denudation prepare a dish containing 100 µlHyaluronidase and 3-5 100 µldrops of Holding or ICSI Medium and denudate 5 ooyctes per time by placing them in Hyaluronidase drop for 30-45 seconds. Transfer with a denudation pipette oocytes to the ICSI drops and denudate further by pipetting to remove remaining cumulus cells. Consider to work under oil overlay if procedure takes longer than a few minutes or several rounds of oocytes will be processed through the same dish. Do not incubate as pH will get too low.
- Do a final rinse through 500 µl wells of ICSI medium.
- Assess oocytes for extrusion of polar body in this dish. Oocytes with a visible polar body are ready for ICSI – if no polar body is detected place them back into freshly equilibrated IVM medium and place them in the incubator again for further maturation. Rinse once through IVM medium in order not to dilute with ICSI medium for the continued maturation. Check every 1-2 hours for extrusion of polar body.

## Semen Preparation

For Semen preparation there are two options Available “Semen Centrifugation and Swim Up, or Sperm Gradient”.

**Semen Centrifugation and Swim Up**

- Prepare a thermos container with 37 °C warm water.
- Label as many 15 ml centrifuge tubes as semen straws to be thawed.
- Add 8 ml Semen Wash Medium per 15 ml centrifuge tube – keep warm - **NOT** $CO_2$ equilibrated
- Verify name of stallion and quickly remove desired number of semen straws from $LN_2$ and place into the thermos container.
- If a straw needs to be cut in half or a quarter–make sure that the straw is always kept under liquid nitrogen and leave the name of the stallion on the unused half
- Dry straw(s) with sterile gauze and place in LAF bench or on a warm surface at 35 °C stage
- Cut the end of the straw opposite from the cotton plug and place the straw just below Semen Wash Medium surface inside inside the centrifuge tube.
- Cut the other end of the straw off (the cotton plug) and allow semen to flow into the 8 ml Semen Wash Medium tube.
- Place a drop of semen from the straw on a warm microscope slide, add coverslip and check the motility immediately.
- Centrifuge using a fixed bucket and non-refrigerated centrifuge for 5 minutes at 328 xg (RCF).



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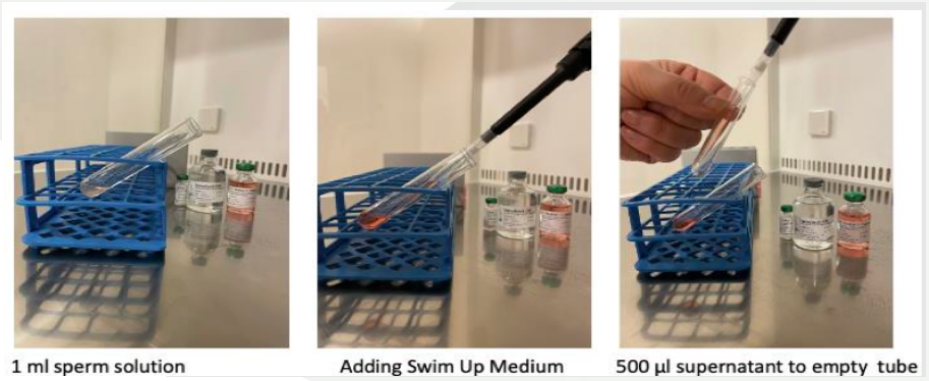
***Do not confuse with RPM, which is rounds per minutes, and depends on length of rotor arm, whereas RCF is the relative centrifugal force (g)***

- Remove supernatant (and keep it in a separate tube in case final sperm concentration is too low) with a 10 ml pipette and leave ~600 µl of the suspension and re-suspend pellet.

***Remove supernatant immediately because swim-up occurs as soon as centrifugation stops***

- Add 6 -8 ml (depending on volume and cryoprotectiva of the semen) pre-warmed Semen Wash Medium to the 600 µl sperm solution and centrifuge for 5 minutes at 328 xg (RCF) to wash semen a second time
- Remove supernatant as described above and leave 1 ml this time and re-suspend pellet again to the final sperm solution. Consider removing the semen pellet instead with a 1 ml pipette into an empty preheated centrifuge tube.
- Either way: work extremely fast as swim up occurs immediately after centrifugation.
- Place the centrifuge tube with 1 ml sperm solution at an angle of 45 degrees
- Add carefully 1 ml Swim Up Medium to overlay the sperm solution.
- Allow for swim up for ½ hour in the laminar flow hood
- Remove 500 µl supernatant from the sperm swim up solution to an empty tube
- Prepare a tube with 95 µl Swim Up Medium (estimating to add 1-5 µl sperm solution)
- Count and determine the sperm solution concentration needed to add to the 95 µl Swim-Up Medium tube in order to have 500.000 in 1 ml final solution used for ICSI.



1 ml sperm solution | Adding Swim Up Medium | 500 µl supernatant to empty tube

• The optimal sperm concentration is 500.000 spermatozoa/ml for ICSI

See Appendix 1. on how to calculate the dilution of your sperm suspension to obtain 500.000 spermatozoa/ml.



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# Sperm Gradient System

## Fresh Semen Samples

1. Transfer 2.5 ml Sperm Gradient Medium Upper Layer 45 % into a sterile disposable centrifuge tube.
2. Using a syringe with needle, place 2.5 ml Sperm Gradient Medium Lower Layer 90 % under the Upper layer. Take care that the two layers are distinctly separated. This is done by placing the tip of the needle on the bottom of the test tube and slowly dispensing the Lower layer. This two-layer gradient is stable for up to two hours.
3. Gently place up to 2.5 ml of liquefied semen onto the Upper layer using a transfer pipette or syringe. Do not use a higher volume than the volume of the individual gradient layers.
4. Centrifuge for 15 to 18 minutes at 350 g to 400 g. When this centrifugation is completed, you may not be able to visibly see a pellet. If so, it is essential to continue the procedure with a second centrifugation of 3 to 5 minutes.
5. Remove supernatant down to the pellet.
6. Using a syringe, add 2-3 ml Semen Wash Medium and resuspend the pellet.
7. Centrifuge for 8 to 10 minutes at 300 g. Higher sperm concentration will require a maximum of 10 minutes centrifugation to ensure a complete and thorough sperm wash.
8. Remove supernatant down to the pellet and repeat steps 6 and 7.
9. If samples do not liquefy and therefore do not pass through the layers, increasing the centrifugal force up to, but no more than, 500 g will help to separate the sperm.

## Frozen Semen Samples

1. Transfer 1 ml Sperm Gradient Medium Upper Layer 45 % into a sterile disposable centrifuge tube.
2. Using a syringe with needle, place 1 ml Sperm Gradient Medium Lower Layer 90 % under the Upper layer. Take care that the two layers are distinctly separated. This is done by placing the tip of the needle on the bottom of the test tube and slowly dispensing the Lower layer. This two-layer gradient is stable for up to two hours.
3. Gently place the thawed semen sample onto the Upper layer using a transfer pipette or syringe (0.5 ml maximum).
4. Centrifuge for 15-20 minutes at 350 g.
5. Remove supernatant down to no less than the 0.5 ml mark above the pellet.
6. Using a syringe, add 2-3 ml Semen Wash Medium and resuspend the pellet.
7. Centrifuge for 8 to 10 minutes at 300 g.
8. Remove supernatant down to the pellet and repeat steps 6 and 7.
9. Remove supernatant and replace with a suitable volume of appropriate medium.



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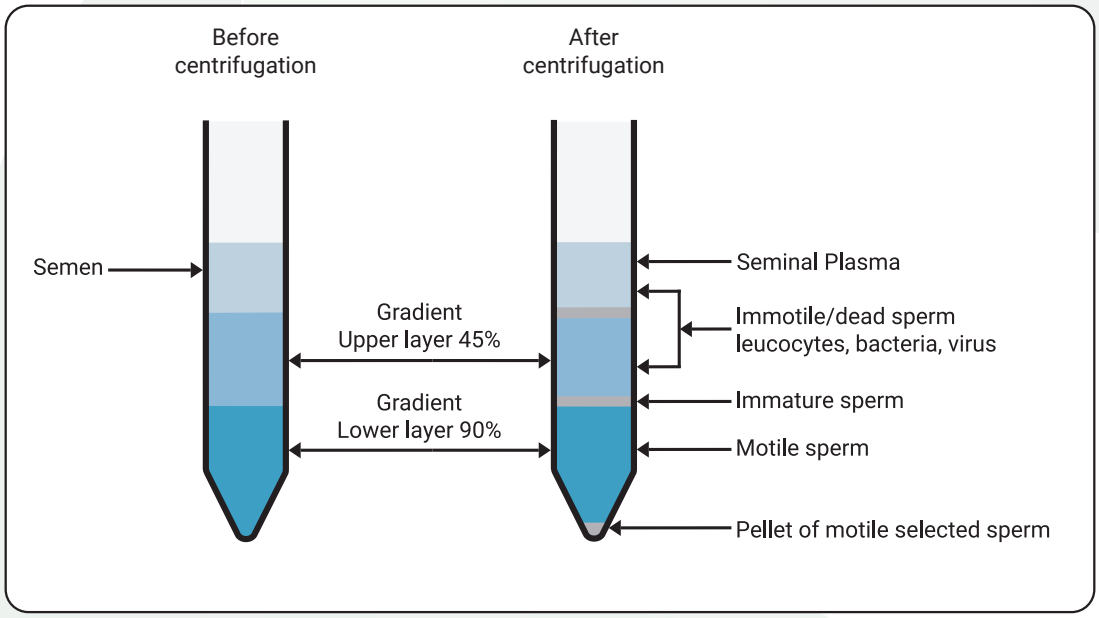
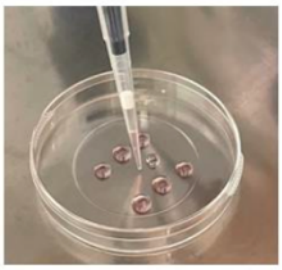
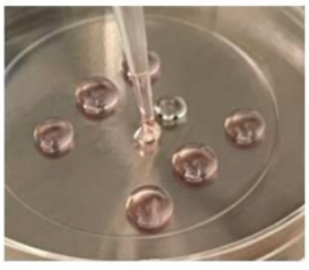
# Stroebech Media Sperm Gradient System

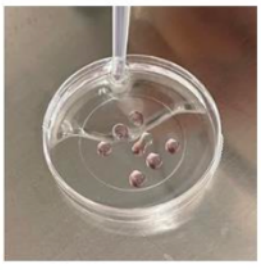


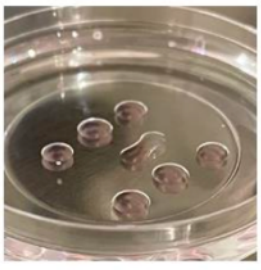

## ICSI Dish Preparation

- Place 5 µl droplets of ICSI medium in lines or a circle in a 50x9 mm ICSI dish. Place one 5 µl droplet of PVP medium in the centre of the dish
- Place the 5 µl sperm solution close to the PVP drop – connect the two drops by placing the pipette in the sperm solution drop and gently move it towards the PVP drop and remove the pipette as soon as the 2 drops are connected. (The motile sperm will swim into the PVP drop)
- Overlay with Stroebech Heavy Oil


6 ICSI drops and PVP drop


adding sperm solution and bridging


adding oil overlay


bridged sperm solution with PVP



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# Setting up and performing ICSI

## ICSI Micromanipulator

The key to good practice while performing ICSI is to ensure that the ICSI micromanipulator is set up correctly with care and precision prior to commencing any manipulations. Problems will be encountered during the ICSI procedure if the initial setup of the micromanipulator is incorrect.

- The holding pipette should be fitted and positioned first and can then be used as a guide for aligning the injection pipette. Use an empty dish marked with a cross for focus and alignment of pipettes.
- Get the stage surface into focus, and then move the focus slightly above the surface of the dish.
- Bring the holding pipette briefly to the optical axis before looking into the microscope, and then move the pipette tip towards the opposite side across the optical axis.
- Begin to move the Y-axis of the course manipulator until a shadow of the pipette can be seen the field of view. If the pipette is angled, the pipette holder can be rotated so it is straight.
- Once the holding pipette is in focus, the process can be repeated for the injection pipette. However, the injection pipette is also used for the immobilisation of sperm prior to injection and so must be set at a slightly deeper angle than horizontal (see figure below).
- The pipette's tip can be moved towards the tip of the holding pipette, and correction of any angling of the pipette can also be corrected here, once the pipette is in focus and in the field of view.
- Once both pipettes are in focus, the initial pipette arrangement is complete.

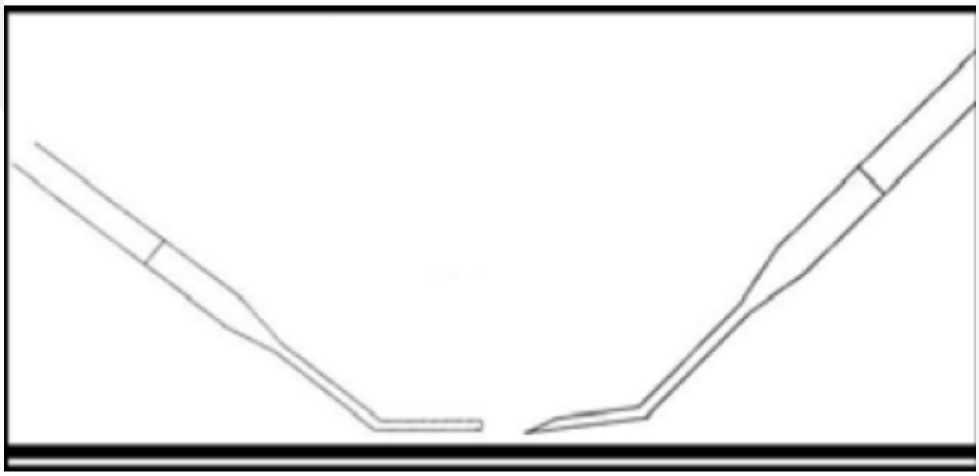

Image Illustrating the complete alignment of both injection and holding pipette.
Take note of the deeper angle on the injection pipette.



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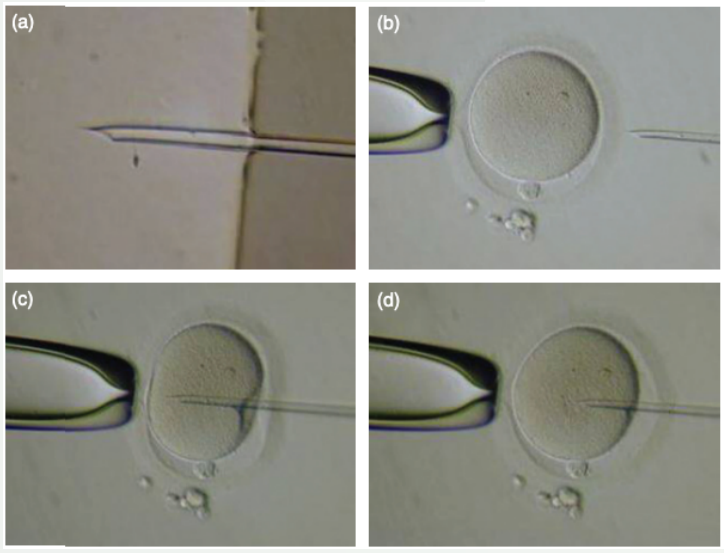
## INTRACYTOPLASMIC SPERM INJECTION (ICSI)

- Place the ICSI dish on the ICSI micromanipulator stage. Make sure the surface is not too hot – do not rely on display alone, but double check the temperature. Use a 10-34 µm ID, inner diameter, holding pipette and a 5.5 µm ID injection pipette.
  **(Note: some human sperm injection pipettes are too narrow for stallion sperm)**
- Identify regular looking, progressively motile sperm, if it is immobile touch the tail, it will most likely move. It had to be motile to swim into the PVP.
- Cut several times across the tail with the pipette and aspirate it into the injection pipette,
- Move the injection pipette with the sperm carefully through the oil into the ICSI drop containing the oocyte(s).




The ICSI procedure – (a) The immobilisation of sperm by breaking the tail and trapping it between the injection pipette and bottom of the dish. The sperm is then taken tail first into the pipette. (b) The oocyte is held in place by the holding pipette with the polar body at 6 (or 12) o'clock. Notice the injection pipette is focused in the same focal plane as the oolemma. (c) The introduction of the injection pipette at the 3 o'clock position, which induces the invagination of the oolemma just as the injection pipette advances to the centre of the oocyte. (d) The sperm is then injected into the centre of the oocyte and the needle is gently withdrawn.

- Position the injection pipette at 3 o'clock and prior to injecting the sperm aspirate gently a small amount of ooplasm to ensure the injection pipette has penetrated the oolemma and is inside the oocyte.
- Inject the ooplasm with the sperm into the oocyte.
- Gently remove the pipette and check the sperm stays in the oocyte.

## IN VITRO CULTURE (IVC) – STEP 1

- Rinse the injected oocytes in $CO_2$ equilibrated IVC Medium 1- Cleavage/One-Step IVC Medium and transfer to the final IVC Medium 1- Cleavage/One-Step IVC Medium well (500 µl with a 400 µl Oil overlay) for culture.
- Incubate at 38.2 °C until day 4-5.



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# DAY 4-5: Blastocyst Culture

**Media to be used**

Equine IVC Medium 2-Blastocyst, or Equine IVC One-Step Medium and Heavy Oil.

**Media & Dish Preparation**

- Prepare an IVC 4WP with 500 µl wells IVC Medium with a 400 µl Oil overlay. Prepare one well for each IVC Medium. Keep the embryos close together in a group when you transfer them gently to the next IVC Medium. Do not separate them but keep them together and minimize disturbance.

**IN VITRO CULTURE (IVC) – STEP 2**

- Transfer the embryos gently from the IVC Medium 1- Cleavage to the IVC Medium 2 -Blastocyst 4WP or from One-Step IVC Medium to new freshly $CO_2$ equilibrate One-Step IVC Medium
- Note cleavage stage
- Evaluate the embryos daily from day 7 to day 10 post-ICSI for blastocyst development.

***Morphologic evaluation of equine blastocysts is challenging, consider practicing by staining with Hoechst to determine whether a blastocyst in fact has been obtained. For photos of blastocysts see: Effects of in Vitro Production on Horse Embryo Morphology, Cytoskeletal Characteristics, and Blastocyst Capsule Formation, Biology of Reproduction https://doi.org/10.1095/biolreprod.103.018515***

# DAY 7-10: POST IVC

Cleavage can be checked 48 hours after – but embryos are best left alone until after morula compaction day 4-5.

- Transfer at day 7 -10 BUT recipient should only be at day 3-4
- Use Holding Medium for immediate transfer
- If transporting the embryos to recipients preferably use vials and load at the transfer site. Never transport embryos in straws

***ICSI embryos are NOT in vivo embryos. They are more fragile and sensitive to pH, temperature and osmolarity fluctuations.***



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# APPENDIX 1

## Semen Calculation and Dilution

Take 10 µl of the sperm solution and count the number of sperm in a Makler Chamber.
The number of sperm counted in 100 squares must be divided by 10 (in Makler Chamber), to give the actual sperm concentration $x10^6$/ml.

The optimal concentration for ICSI is 0,5 $x10^6$/ml in 5 µl drops

## EXAMPLE:

If the number of sperm I 100 square is 100, you divide it with 10 and end up with a final concentration at 10 $x10^6$ sperm/ml.
You want 100 µl suspension with a final concentration of 0,5$x10^6$ sperm/ml for fertilization:

$$\textit{Dilution factor} = \frac{\textit{Number of sperm/ml}}{\textit{0,5 x } 10^6 \textit{ sperm/ml}}$$

$$\textit{Dilution factor} = \frac{\textit{10 x } 10^6 \textit{ sperm/ml}}{\textit{0,5 x } 10^6 \textit{ sperm/ml}} = 20$$

*Final volume / dilution factor = 100 µl / 20 = 5 µl*

*Final dilution: 5 µl suspension + 95 µl Swim up medium*



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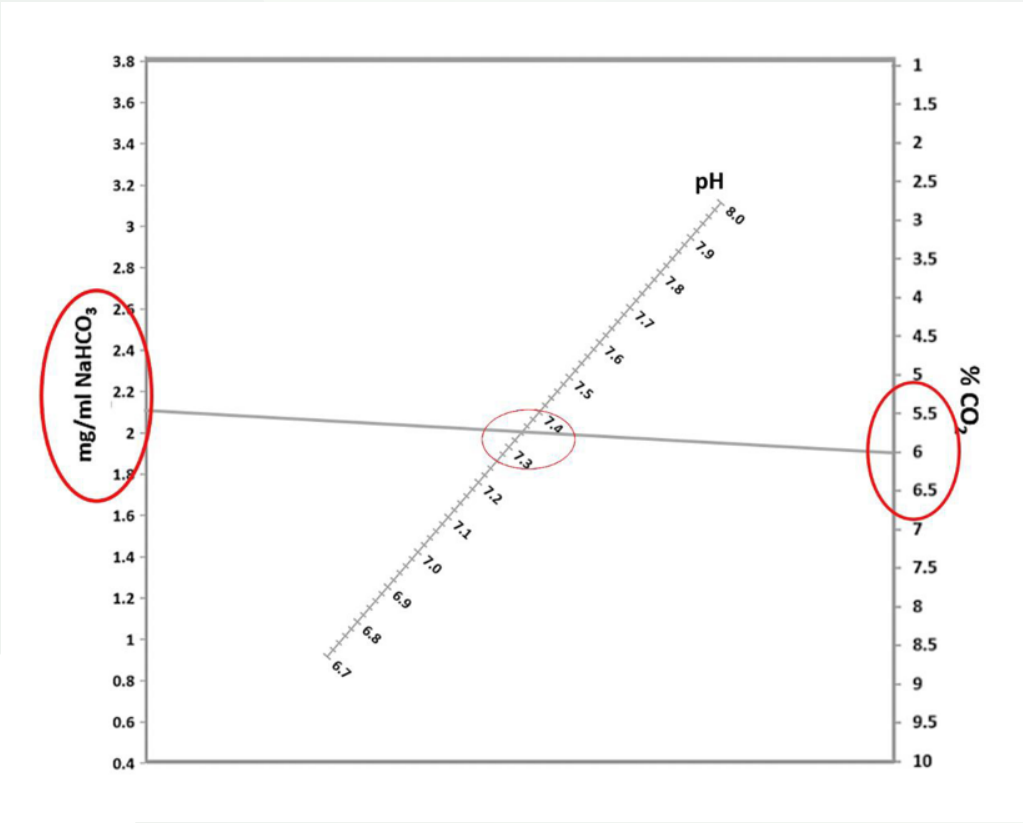
# APPENDIX 2

## Correlation between Bicarbonate and $CO_2$ Concentrations and the effect on pH



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# APPENDIX 3

**Correction table to maintain the same partial pressure in incubator of $CO_2$ and $O_2$ as at sea level**

| Altitude above Sea Level (Meter) | Altitude above Sea Level (Atm) at 39 degrees | $CO_2$ and $O_2$ |
|---|---|---|
| 0 | 1 | 6 |
| 100 | 0,99 | 6,1 |
| 300 | 0,97 | 6,2 |
| 500 | 0,95 | 6,3 |
| 700 | 0,93 | 6,5 |
| 900 | 0,91 | 6,6 |
| 1100 | 0,89 | 6,7 |
| 1300 | 0,87 | 6,9 |
| 1500 | 0,85 | 7,1 |
| 1700 | 0,83 | 7,2 |
| 1900 | 0,81 | 7,4 |
| 2100 | 0,79 | 7,6 |
| 2300 | 0,77 | 7,8 |
| 2500 | 0,76 | 7,9 |
| 2700 | 0,74 | 8,1 |
| 2900 | 0,72 | 8,3 |
| 3100 | 0,7 | 8,6 |
| 3300 | 0,69 | 8,7 |
| 3500 | 0,67 | 9 |
| 3700 | 0,66 | 9,1 |
| 3900 | 0,64 | 9,4 |

## EXAMPLE:

Laboratory is **at 1500 m above** sea level where the partial pressure is 0,85 atm. The desired $CO_2$ concentration is 6 % then you calculate 6/0,85 atm = 7,1 and your incubator setting **should be 7.1 % $CO_2$** in order to have 6 % $CO_2$ inside the incubator



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